EFFECTS OF PHOSPHOLIPID SURFACE CHARGE ON ION CONDUCTION IN THE K^+ CHANNEL OF SARCOPLASMIC RETICULUM

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ABSTRACT Single-channel K^+ currents through sarcoplasmic reticulum K^+ channels were compared after reconstitution into planar bilayers formed from neutral or negatively charged phospholipids. In neutral bilayers, the channel conductance saturates with K^+ concentration according to a rectangular hyperbola, with half-saturation at 40 mM K^+, and maximum conductance of 220 pS. In negatively charged bilayers (70% phosphatidylserine/ 30% phosphatidylethanolamine), the conductance is, at a given K^+ concentration, higher than in neutral bilayers. This effect of negative surface charge is increasingly pronounced at lower ionic strength. The maximum conductance at high K^+ approaches 220 pS in negative bilayers, and the channel's ionic selectivity is unaffected by lipid charge. The divalent channel blocker "bisQ11" causes discrete blocking events in both neutral and negatively charged bilayers; the apparent rate constant of blocking is sensitive to surface charge, while the unblocking rate constant is largely unaffected. Bilayers containing a positively charged phosphatidylcholine analogue led to K^+ conductances lower than those seen in neutral bilayers. The results are consistent with a simple mechanism in which the local K^+ concentration sensed by the channel's entryway is determined by both the bulk K^+ concentration and the bulk lipid surface potential, as given by the Gouy-Chapman model of the electrified interface. To be described by this approach, the channel's entryway must be assumed to be located 1–2 nm away from the lipid surface, on both sides of the membrane.

INTRODUCTION

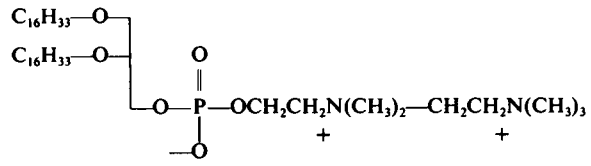
An unusually intractable problem in the study of ion channels is the extent to which membrane surface charge is involved in the operation of these membrane-spanning proteins. The idea that such an involvement might be physiologically relevant was first introduced by Frankenhauser and Hodgkin (1957), who showed that removal of Ca^{++} ion from the extracellular medium shifts the squid axon Na^+ channel's activation curve towards a more negative transmembrane voltage. The interpretation was offered that Ca^{++} binds to the external membrane surface and alters the surface potential, thus "polarizing" the intramembrane electric field experienced at the channel's voltage sensor. Later, Gilbert and Ehrenstein (1969) pointed out that qualitatively similar effects could arise from Ca^{++}-induced charge screening rather than from specific binding. More recent studies with giant axons have employed divalent cations to assess the degree to which the surface potential is sensed by both the gating and conduction mechanisms of Na^+ and K^+ channels (Begenisich, 1975; Fohlmeister and Adelman, 1982). Both these approaches concluded that the ion conduction process is rather insensitive to surface potential, as if the channel's "mouth" were far removed from the bulk lipid surface. However, it is difficult to conclude this rigorously, since divalent cations may interact with channel proteins in ways other than by screening membrane surface charge.

This report attacks the question of lipid surface charge effects on ion conduction through an integral membrane channel, the K^+ channel of sarcoplasmic reticulum (SR). We exploit a capability not available in the above work on axon channels—the ability to directly measure K^+ currents through single channels in membranes of defined and controllable lipid compositions. It is thus possible to change the surface potential directly by reconstituting channels into membranes of differing surface charge densities and to observe the resulting differences in single-channel currents. It is shown that this channel's K^+ conduction process does indeed respond to the lipid surface charge, and that the effects can be related quantitatively to electrostatic double-layer theory by assuming that the channel's ion entryway is removed from the bulk lipid surface by a distance of 1–2 nm.

MATERIALS AND METHODS

The preparation of SR vesicles was carried out as previously described (Miller and Rosenberg, 1979). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were prepared from egg yolk (Labarca et al., 1980). Phosphatidylserine (PS) was purchased from Avanti Biochemicals, Inc. (Birmingham, AL), and was not purified further. A positively charged PC analogue, 1,2-dihexadecylphosphatidyldicholine (DHPDC),

was generously donated by Dr. G. Boheim; its structure is shown below:



All aqueous solutions contained 5 mM MOPS (morpholinopropanesulfonic acid), 0.5 mM EDTA, the appropriate concentration of K^+ gluconate, and 2.5 mM KOH to adjust the pH to 7.1. The only cation present in these solutions (other than H^+) was K^+. In most experiments, neutral membranes were composed of 80 PE/20% PC, while negatively charged membranes were formed from 70 PS/30% PE. It was necessary to use these lipid mixtures, rather than the pure lipids, because of constraints on the reconstitution of the channels. In several experiments, pure PE bilayers were used, with results identical to those obtained in PE/PC bilayers. It was not possible to observe SR K^+ channels reliably in pure PS bilayers. Some experiments employed the channel blocker "bisQ11," an undecyl analogue of decamethonium, which was synthesized as previously described (Miller, 1982).

Membranes were formed by apposition of two phospholipid monolayers spread on the buffer solution by the technique of Montal and Mueller (1972). The chambers were made of Teflon divided by a 13-μm thick Teflon partition. A hole (50–200 μm diam) was made in the partition by bringing a hot platinum wire close to the surface of the film and quickly withdrawing it as the film began to melt. Before spreading, the lipids were dried down and redissolved in pentane to a concentration of 20 mM. Monolayers were spread by dropping 10 μl of the appropriate lipid solution on the surface of the electrolyte. After allowing the pentane to evaporate, the solutions in both compartments were raised over the top of the hole.

SR vesicles were fused with the bilayer by making the *cis* aqueous solution (the side to which the vesicles are added) 200 mM hyperosmotic to the *trans* solution, using either K^+-gluconate or urea. This condition was an absolute requirement for insertion of channels into these "solvent-free" bilayers (Labarca et al., 1980). SR vesicles (1–10 μg/ml) were added to the *cis* side, in the presence of 0.5 mM $CaCl_2$, also on the *cis* side. Immediately after a fusion event was observed (as an abrupt increase in bilayer conductance), 1 mM EDTA was added to the *cis* side, which was then extensively perfused with the same solution contained in the opposite chamber. In this way, all measurements were performed with symmetrical ionic compositions, and in the absence of excess SR vesicles or divalent cations.

Single channel fluctuations were recorded at holding potentials between −60mV and +60 mV, and were stored on FM tape and subsequently analyzed by hand or with a MINC 11/23 computer. Experiments measuring the mean blocked and unblocked times of the open channel in the presence of the flickering blocker bisQ11 were recorded at 2 KHz time resolution and were sampled at one point per ms by the computer. Typically, 500–2,000 transitions were analyzed for the calculation of mean open and blocked times, as described (Miller, 1982).

Surface charge density was estimated in two ways, both involving measurement of the surface potential, ψ_0. The first is the nonactin method of McLaughlin et al. (1970)

$$\psi_0 = -RT/F \ln(G^*/G_0) \quad (1)$$

where G^* is the membrane conductance induced by nonactin before the addition of inert electrolyte and G_0 is the membrane conductance after the surface potential has been neutralized by adding high concentrations of LiCl or $CaCl_2$. A bilayer was formed using the lipid mixture to be tested, in the presence of a low concentration of K-gluconate (0.2 mM–100 mM), and nonactin was added with stirring in both chambers until a stable conductance of 10^{-3} to 10^{-4} S/cm^2 was reached. LiCl was then added to both sides in aliquots until no further change in conductance was seen. This conductance value was taken to be the neutral membrane conductance. Surface charge density, σ, was calculated using the Gouy-Chapman-Stern theory for uni-univalent electrolytes (McLaughlin, 1977)

$$\sinh(F\psi_0/2RT) = \sigma/[1 + K_a K_b \exp(-F\psi_0/RT)][8\epsilon\epsilon_0 RTK_b]^{1/2} \quad (2)$$

where K_b is the bulk K^+ concentration, ϵ and ϵ_0 are the dielectric constant and the permittivity of free space, F, R, and T have their usual meanings, and K_a, the association constant of K^+, is taken to be 0.15 M^{-1} (Eisenberg et al., 1979).

Alternatively, surface potential was measured as the potential across the air-buffer interface with a polonium air electrode (Ohki and Sauve, 1978). The electrode over a lipid monolayer measures the sum of the surface and dipole potentials of the lipid. Raising the ionic strength changes the surface potential but does not affect the dipole potential; hence the difference between the potentials measured at various low (2–200 mM) and high concentrations is taken to be the surface potential in the low salt solution. Charge density is then calculated as above.

Table I shows that the surface charge densities obtained from the two methods agree well with each other, and that in negative bilayers they also agree with the values expected from the molecular area for PS in a condensed monolayer. The results also show that the PE/PC bilayers carry a slight negative charge, which may be accounted for by the fraction of unprotonated ethanolamine at this pH value; this amount of charge is entirely negligible for the effects to be addressed here, and we will refer to the PE/PC bilayers as "neutral." The bilayers containing the positively charged PC analogue DHPDC display a surface charge density only half of that expected from the monolayer composition. The charge densities reported in Table I were measured in the absence of SR vesicles. In several experiments, similar values were obtained in bilayers into which vesicles had previously been fused (see Table I). It is expected that the presence of SR should not affect the charge density, since the fraction of lipid in a bilayer contributed by a single 100 nm radius vesicle is $<10^{-5}$.

The local concentration of K^+ at a distance x normal to the membrane surface was assumed to follow a Bolzmann distribution in the electrostatic potential field

$$K(x) = K_b \exp\{-F\psi(x)/RT\} \quad (3)$$

where $K(x)$ and $\psi(x)$ are the K^+ concentration and potential at x. The

TABLE I
DETERMINATION OF SURFACE CHARGE DENSITY

Lipid	Method		Average	%
	Monolayer	Nonactin		
80% PE/20% PC	<0.04	0.08 ± 0.02	0.06 ± 0.03*	4
70% PS/30% PE	0.85 ± 0.1	0.96 ± 0.2	0.9 ± 0.2*	64
25% DHPDC/ 75% PE	0.19 ± 0.07	0.18 ± 0.07	0.19 ± 0.05	13

Measurements of surface charge are reported as charges/nm^2. With both techniques the surface potential at a given electrolyte concentration was considered to be neutralized at high ionic strength (see Methods). Charge density was then calculated according to Eq. 2. Data represent the mean and standard error of at least two determinations at 2–5 different electrolyte concentrations. The final column reports the apparent percentage of charged lipid in the bilayer calculated from the measured charge density, assuming a molecular area of 0.7 nm^2 per phospholipid (Loosely-Millman et al., 1982).

*Nonactin experiments performed on bilayers into which SR vesicles had previously been fused gave values within the reported range.

variation of potential with distance, in turn, is given by Gouy-Chapman-Stern theory (McLaughlin, 1977)

$$\psi(x) = 2RT/F \cdot \ln([1 + \alpha\exp(-\kappa x)]/[1 - \alpha\exp(-\kappa x)]) \quad (4a)$$

where κ, the reciprocal debye length, is

$$\kappa = (2F^2K_b/\epsilon\epsilon_0RT)^{1/2}, \quad (4b)$$

and

$$\alpha = [\exp(F\psi_0/2RT) - 1]/[\exp(F\psi_0/2RT) + 1]. \quad (4c)$$

The surface potential ψ_0 in Eq. 4c was taken from the measured charge density and the bulk salt concentration, as in Eq. 2. The K^+ concentration at a fixed distance from the membrane was calculated as a function of bulk K^+ by an iterative procedure using Eqs. 2–4.

RESULTS

This study embarks from the experimental fact illustrated in Fig. 1. Single-channel currents through K^+ channels from SR vary with the surface charge of the planar bilayer membranes into which they are incorporated. In negatively charged membranes (70% PS/30% PE), the channel conductance is higher than in neutral membranes (80% PE/20% PC). The effect of the PS-containing membranes in raising the K^+ conductance is much more dramatic in low K^+ solutions (22.5 mM) than in high K^+ solutions (400 mM). A simple idea which would explain this effect qualitatively is to assume that the local K^+ concentration actually sensed by the channel protein is near enough to the lipid surface to be determined by the membrane surface potential. Thus, in a neutral membrane of zero surface potential, the local K^+ concentration would be equal to the bulk K^+ concentration; in a negatively charged membrane the local concentration would be substantially higher than in the bulk solution, and the K^+ conductance would be correspondingly higher. The remainder of this report will examine whether this suggestion stands up to quantitative tests.

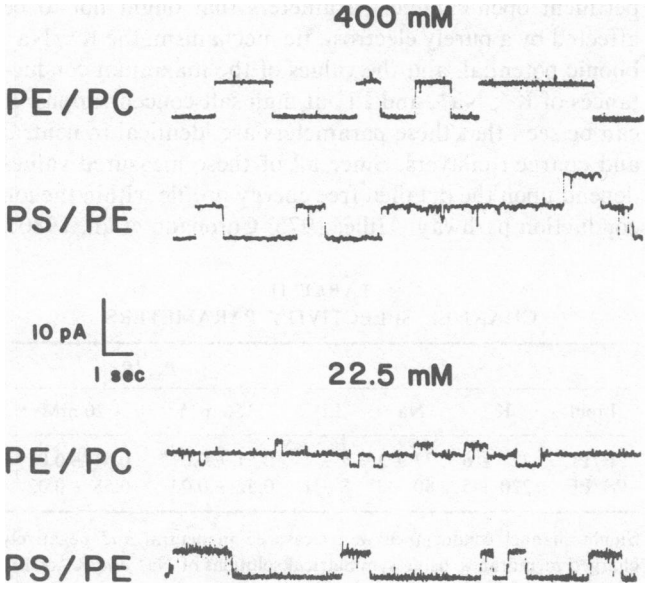


FIGURE 1 Single-channel current fluctuations in neutral and negatively charged membranes. SR vesicles were fused as described in Methods with symmetrical bilayers formed from monolayers composed of charged (PS/PE) or neutral (PE/PC) lipids. The aqueous solutions contained the indicated K^+ concentration. The holding potential in all cases was −30 mV. In all recordings presented here, an upward deflection represents increased conductance.

Variation of Channel Conductance With K^+ Concentration

The variation in channel conductance, γ, with K^+ concentration was determined in solutions in which K^+ is the only cation, i.e., in varying ionic strength. In neutral bilayers, the conductance follows simple "Michaelis-Menten" saturation behavior (Fig. 2 *A*)

$$\gamma = \gamma_{max} K_b/K_m/[1 + K_b/K_m] \quad (5)$$

where γ_{max} is the maximum conductance, and K_m the apparent dissociation constant. This behavior has been

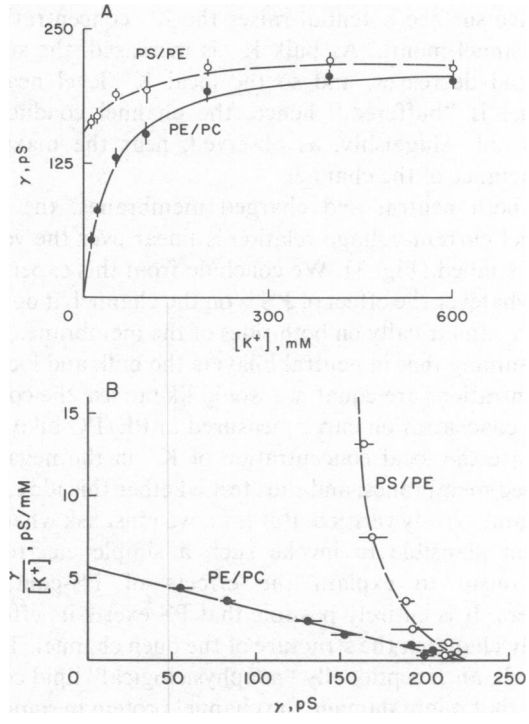


FIGURE 2 Single-channel conductance as a function of K^+ concentration. Single-channel conductances were measured at several voltages and the indicated K^+ concentrations as in Fig. 1. *A*, closed circles (•) represent the conductances obtained in PE/PC bilayers and are well fit by a rectangular hyberbola with dissociation constant of 40 mM and maximum conductance of 220 pS. Open circles (○) are the data from PS/PE membranes. The curve through these points was drawn by eye, and carries no theoretical connotation. Points represent SEM of at least 30 determinations in 2–5 membranes. *B*, scatchard plots of the same data, drawn so that both sets of data approach 220 pS at high K^+ concentrations.

documented in detail previously (Coronado et al., 1980) and has been used to argue that this channel operates by a single-ion mechanism in which at most one K^+ ion can occupy the channel at a time. The Scatchard plot of these data, taken down to 12.5 mM K^+, shows that this behavior is followed well over the entire concentration range tested here (Fig 2 *B*). In negatively charged, PS-containing bilayers, however, a dramatically different result is obtained. At high K^+ concentrations, the conductance is near to the γ_{max} seen in the neutral membrane; but as K^+ concentration is lowered, the conductance remains high. The departure of the conductance in the negatively charged membranes from that in the neutral membranes becomes increasingly pronounced at low K^+, i.e., at lower ionic strength. The Scatchard plot of these data (Fig 2 *B*) emphasizes the differences in the two membranes, and also shows that a fit to a Michaelis-Menten law cannot in any way be made tenable in the negatively charged membranes.

These results can be qualitatively explained by assuming that the higher conductance in the negatively charged membranes is due to an electrostatic effect of the charged surface: that at low K^+ (and low ionic strength), a high negative surface potential raises the K^+ concentration at the channel mouth. As bulk K^+ is increased, the surface potential decreases, and so the local K^+ level near the channel is "buffered;" hence, the channel conductance varies only sluggishly, as observed, near the maximum conductance of the channel.

In both neutral and charged membranes, the open-channel current-voltage relation is linear over the voltage range studied (Fig. 3). We conclude from this experiment that whatever the effect of PS is on the channel, it operates quite symmetrically on both sides of the membrane.

Assuming that in neutral bilayers the bulk and local K^+ concentrations are equal, we would like to use the conductance-concentration curve measured in PE/PC bilayers to calibrate the local concentration of K^+ in the negatively charged membranes, and thus test whether this idea might be quantitatively verified. But first, we must ask whether it is even plausible to invoke such a simple electrostatic mechanism to explain the effects of PS-containing bilayers. It is entirely possible that PS exerts its effect by merely changing the structure of the open channel. This is, after all, an exceptionally "nonphysiological" lipid composition that might damage the channel protein in some way. Below we will argue, on the basis of three lines of evidence, that PS does influence the channel conductance by an electrostatic mechanism, and not by altering the basic structure of the membrane protein.

Channel Conduction Parameters

The conduction and selectivity properties of this channel have been previously studied in some detail in neutral membranes (Coronado et al., 1980). If the effect of PS is to change the channel structure, we might expect some changes in these basic properties. In Table II, we list two pertinent open-channel parameters that ought not to be affected by a purely electrostatic mechanism: the K^+/Na^+ biionic potential, and the values of the maximum conductances of K^+, Na^+, and Li^+ at high salt concentrations. It can be seen that these parameters are identical in neutral and charged bilayers. Since all of these measured values depend upon the detailed free energy profile within the ion conduction pathway (Hille, 1975; Coronado et al., 1980),

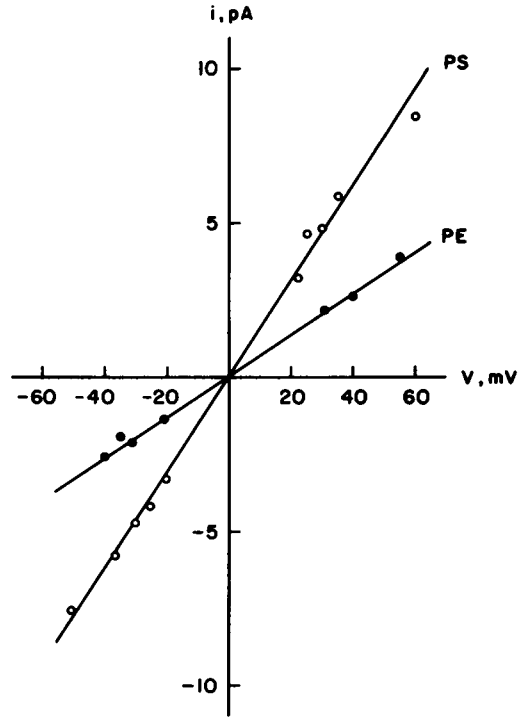


FIGURE 3 Open channel current-voltage curves in neutral and charged membranes. Records of channel current fluctuations in 12.5 mM K^+ gluconate were collected at different holding voltages. Conductances calculated from these data are 160 pS in PS/PE bilayers, and 65 pS in PE/PC bilayers.

TABLE II
CHANNEL SELECTIVITY PARAMETERS

	γ_{max}			P_{Na}/P_K	
Lipid	K^+	Na^+	Li^+	150 mM	20 mM
PE/PC	223 ± 6	77 ± 1	7 ± 1	0.51 ± 0.01*	0.51 ± 0.01*
PS/PE	220 ± 5	80 ± 3	8 ± 1	0.52 ± 0.03	0.58 ± 0.02

Single channel conductance was measured in neutral and negatively charged membranes, using symmetrical solutions of Na^+ and K^+ or Li^+ solutions to obtain the maximum channel conductance, γ_{max}. γ_{max} values in the PE/PC membranes were obtained from Scatchard or double reciprocal plots, while those in PS/PE bilyers were assumed to be equal to the value measured at 400 mM.

Permeability ratios were measured under biionic conditions with K^+ on the *cis* side of the bilayer and Na^+ at the same concentration on the trans side. Permeability ratios were calculated from the biionic potential, V_0, according to: $P_{Na}/P_K = \exp(-FV_0/RT)$.

*Constant between 0.05–0.5M (Coronado et al., 1980).

we conclude that this conducting structure is little affected by the presence of high levels of PS in the bilayer.

Kinetics of Divalent Blockers

It has previously been shown that the SR K^+ channel is blocked from the *trans* side of the membrane by decamethonium and its long-chain analogues (Coronado and Miller, 1980; Miller, 1982). The residence time of such a blocker molecule on the blocking site within the channel's conduction pathway is long-lived enough that discrete, single-molecule blocking kinetics can be observed directly. Fig. 4 compares records, taken from neutral and charged bilayers, of discrete block, or "flickering," induced by the bis-quaternary ammonium blocker, bisQ11 (Miller, 1982).

We wish to make two points from these records. First, the qualitative appearance of the blocking behavior is the same in both types of membranes: the PS-containing bilayers do not alter the essential effect of the blocker's interaction with the channel. The second point to be noted from these experiments is that there are quantitative differences in the blocking kinetics in the different membranes. It can perhaps be discerned from Fig. 4 that the probability of a blocking event, i.e., a downward "flicker," is higher in the PS membrane than in the neutral membrane. On the other hand, the average residence time of the blocker within the channel, τ_b, is not obviously changed. These results are summarized in Table III. Under identical conditions of applied voltage and bisQ11 concentration, the mean open time, τ_0, is shorter in negative than in neutral bilayers; the mean block time, τ_b, is not significantly affected.

These results are expected by a purely electrostatic effect of PS. Since the blocking site lies deep within the channel protein (Miller, 1982), the mean residence time should not be much affected by the lipid surface charge. However, the mean open time is a measure of the second-order "on" (rate of blocking), and is thus inversely related to the concentration of blocker sensed by the channel. This concentration, in turn, is dependent on the surface potential, just as is the K^+ concentration. Furthermore, because this blocker is divalent, the electrostatic effect should be greater in raising its local concentration than it is in raising that of the monovalent K^+ ion. Thus, from the measured effect of PS on the K^+ conductance, the electrostatic mechanism predicts the effect of PS on the mean open time of bisQ11 flickering.

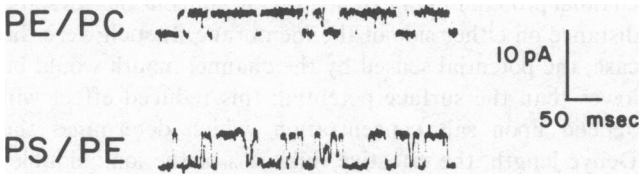


FIGURE 4 Single-channel block by bisQ11. Recordings of single-channel fluctuations in 22.5 mM K^+ and 5μM bisQ11 were collected in neutral or charged bilayers. The figure displays segments of such records within a channel opening. Fluctuations represent discrete blocking and unblocking events of the open channel by bisQ11. Holding potential in both cases was −30mV. Note the higher channel conductance and the enhanced probability of a blocking event in the negatively charged PS/PE bilayers.

TABLE III
EFFECT OF BILAYER CHARGE ON BisQ11 BLOCK

Lipid	Raw Data τ_o	τ_b
	ms	*ms*
Neutral	17 ± 1	7.4 ± 0.5
Charged	6.8 ± 1	8.7 ± 1

Ratios (Charged/Neutral)

	K_{loc}	τ_o	τ_b	B_{loc}
Measured	2.8	0.40 ± 0.06	1.2 ± 0.1	4.4
Predicted	—	0.24–0.44	1.0	4.1–7.2

Mean open and blocked times, τ_o and τ_b, were measured as in Fig. 4. in PS/PE "charged" or PE/PC "neutral" bilayers, using 22.5 mM K^+-gluconate solutions containing 5 μM bisQ11. Holding potential was −30 mV. Each value represents the mean ± SE of 4–6 separate analyses, each carried out on segments containing 500–2000 transitions; most of these segments were taken from different membranes.

The *Raw Data* section of the Table shows the values of open and blocked times. The *Ratios* section displays the measured ratios of various parameters in charged vs. neutral bilayers. These include the local K^+ concentration (K_{loc}) sensed at the channel mouth, calculated from the channel conductance ratio by Eq. 5; the open and blocked times; and the local blocker concentration (B_{loc}), calculated from Eq. 8. The row labeled *Predicted* represents the values of these ratios expected from an electrostatic model of enhanced blocking in charged membranes. The range of values for the τ_o and B_{loc} ratios are calculated from Eqs. 10 and 9, assuming that the effective valence of bisQ11 lies between 1.4 and 2.0 (Alvarez et al., 1983).

There are two complications that must be included in the derivation of the expected bisQ11 behavior. First, it is known that the blocking reaction is competitive with K^+, i.e., that

$$\tau_0^{-1} = kB\{1 + K/K_m\}, \tag{6}$$

where k is the second-order rate constant for blocking in the limit of zero K^+, and B and K represent local blocker and K^+ concentrations near the channel mouth (Miller, 1982). We can now write the mean open times in the neutral and charged bilayers, using primed quantities for the latter, and unprimed quantities for the former

$$\begin{aligned} \tau_0'^{-1} &= kB'\{1 + K'/K_m\} \\ \tau_0^{-1} &= kB\{1 + K/K_m\}. \end{aligned} \tag{7}$$

We can now replace the terms in brackets with directly measurable quantities, the K^+ conductances in the neutral and charged bilayers, by use of Eq. 5 (and some subsequent

algebra)

$$\tau'_0/\tau_0 = (B/B')([\gamma_{max} - \gamma]/[\gamma_{max} - \gamma']). \quad (8)$$

An electrostatic mechanism demands that

$$B/B' = (K/K')^n \quad (9)$$

where n is the valence of the blocker. By applying Eq. 5 and performing several algebraic manipulations, we arrive at the final result

$$\tau'_0/\tau_0 = (\gamma/\gamma')^n([\gamma_{max} - \gamma'] / [\gamma_{max} - \gamma])^{n-1} \quad (10)$$

This brings up the second complication, namely that a molecule like bisQ11 will not, near a charged surface, behave like a point-divalent charged particle. Instead, because of its finite size, its effective valence will generally be <2 and >1. A theoretical treatment of this effect has been presented by Carnie and McLaughlin (1983 *a*), and has been experimentally confirmed by Alvarez et al. (1983) for compounds like bisQ11. The conclusion of this work is that we ought to consider the valence of the blocker under our conditions to be between 2 and 1.4. This, then, places limits on the expected values for mean open-time ratios in the two lipids, given the measured conductance ratios. These values are shown in Table III. It is clear that the electrostatic mechanism adequately accounts for the effect of PS on lowering the mean open time in the presence of bisQ11.

Positively Charged Lipid Effect

We have carried out a limited number of experiments in which channels were inserted into bilayers formed from PE mixed with a positively charged PC analogue. These bilayers contained a rather low positive charge density (Table 1), but a significant lowering of the channel's conductance was nevertheless observed, as expected qualitatively by an electrostatic mechanism (Table IV). Thus, in positively charged bilayers, the local K^+ concentration near the channel's mouth appears to be lower than in bulk solution. This result provides independent evidence that the channel conduction mechanism senses the electrostatic potential set up by the bilayer lipid surface charge.

DISCUSSION

The results presented here have been used to argue that the SR K^+ channel's ion conduction mechanism experiences a local electrostatic potential arising from surface charge on the lipid bilayer. We have used three lines of evidence to support an electrostatic effect and to rule out unspecified perturbations of the K^+ channel's structure caused by high surface charge density on the bilayers into which the channels are incorporated. On the basis of these arguments, we conclude that a charged lipid membrane causes the K^+ concentration near the entryway of the channel to differ from the bulk concentration, and that this effect accounts for the variation in channel conductance in bilayers of different lipid compositions.

TABLE IV
SINGLE CHANNEL CONDUCTANCE IN POSITIVELY CHARGED MEMBRANES

	Observed γ		Predicted γ	
	50 mM	100 mM	50 mM	100 mM
	pS	*pS*	*pS*	*pS*
PE/PC	131 ± 7 (16)	152 ± 7 (5)	131	152
PE/DHPDC	117 ± 2 (14)	141 ± 4 (11)	104	143

Observed single channel conductances, γ,pS, were obtained as described in Methods. The predicted conductances in neutral membranes were taken to be the mean of the observed values. Predicted values for a channel protruding 15 Å from a positively charged bilayer were calculated according to Eq. 2, using the measured surface charge density of 0.19 charges/nm^2 and noting that:

$$\gamma/\gamma' = \frac{1 + K_m/[K^+] \exp(F\Psi/RT)}{1 + K_m/[K^+]}.$$

γ is the conductance in the neutral membrane, γ' in the charged membrane, ψ the potential near the mouth of the channel and K_m the previously determined dissociation constant, 0.04M.

Examination of the primary conductance-concentration data (Fig. 2 *A*) shows, however, that the effect of PS is much less than that expected if the channel were sensing the K^+ concentration immediately adjacent to the lipid bilayer surface. In such a case, the entire lipid surface potential would be felt at the channel mouth; in 70% PS bilayers, the surface concentration would be >1 M over the entire range of K^+ concentrations, and the channel would always display its maximum conductance. Indeed, exactly this type of behavior has been observed for the gramicidin A channel (Apell et al., 1979), which is known to open out to the aqueous phases right at the lipid surface. But this is not the case for the SR K^+ channel, as the conductance in PS bilayers is clearly increasing over the range 20 mM–200 mM K^+, and does not actually attain a value of the maximum conductance until ~250 mM K^+.

It is reasonable to propose that an integral membrane channel protein might extend into the aqueous phases some distance on either side of the membrane. If such were the case, the potential sensed by the channel mouth would be lower than the surface potential; this reduced effect will depend upon salt concentration, which determines the Debye length, the effective thickness of the ionic double-layer near the surface. By applying the expanded Gouy-Chapman theory (see Methods), we can calculate the local concentration of K^+ at a given distance out from the lipid surface as a function of bulk K^+ concentration. These calculations, as well as the data of Fig. 2, are shown in Fig. 5. The enhanced conductance of the channel in the PS-containing bilayers can be explained if we assume that the

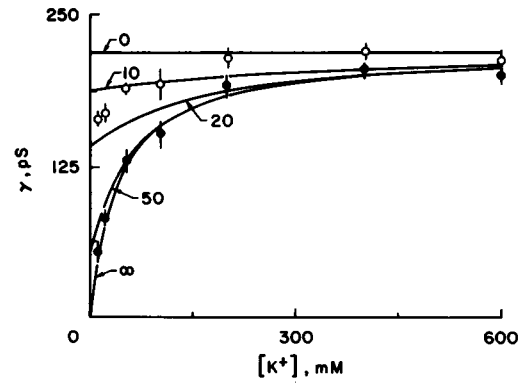


FIGURE 5 Single-channel conductance vs. K^+ concentration at various distances from a negatively charged bilayer. Curves calculated from Eqs. 2–4 represent the expected conductance of the channel protruding the indicated distance, in ångströms, from a bilayer containing the measured surface charge density of 0.93 charges/nm^2. Data plotted are from Fig. 2., where • are the data from PE/PC membranes, and o from PS/PE membranes. Consideration of binding by K^+ ions to the PS headgroups (Eisenberg et al., 1979) does not significantly alter the theoretical curves.

"mouth" is located some 1–2 nm away from the bulk lipid surface. The linear current-voltage relation (Fig. 3) suggests that this degree of isolation from the surface is similar on the two sides of the bilayer.

This characteristic distance of isolation from the surface is also consistent with the lowering of conductance in the positively charged membranes (Table IV). The values of conductance expected in this case were calculated on the basis of a positive local potential 1.5 nm away from the surface, and the agreement with the measured values is tolerable. This result lends further credibility to an electrostatic effect of the lipid surface on the local K^+ concentration near the channel's mouth.

There are several qualifications we should make regarding this picture. It is clear that the data do not precisely fit any of the curves plotted in Fig. 5. We do not expect that they should, since the exact form of these functions depends on the particular geometry of the system. Because we have no information of the structure of the channel's mouth, we cannot expect that it will behave quantitatively as if it protruded into the aqueous solution, opening at a single, well-defined distance from the surface. The purpose of presenting Fig. 5 is to illustrate the approximate scale of distances involved in the surface charge effect. Regardless of the precise geometry, we can say that the channel mouth must be farther removed from the surface than 0.8 nm, and closer than 2.5 nm.

Nor can we say that the channel mouth is removed from the lipid surface by actually protruding into bulk solution. It is also possible that the region of the channel that senses K^+ concentration is insulated laterally from the lipid: a marshmallow with a hole through the middle. Other types of geometrical isolation schemes can easily be conceived. The conclusion remains, however, that a characteristic distance of isolation of 1–2 nm should be involved, regardless of the exact geometry. We are fairly confident in making this assertion since the Gouy-Chapman-Stern theory has been found to be remarkably accurate for the calculation of phospholipid membrane surface potentials (McLaughlin, 1977; Eisenberg et al., 1979; Alvarez et al., 1983; Carnie and McLaughlin, 1983), as well as of Debye lengths in NaCl solutions (Loosley-Millman et al., 1982), though in this latter case, employing force measurement methods, significant deviations from theory were noted in LiCl solutions.

A final conclusion arising from this work is that the SR K^+ channel protein itself presents essentially uncharged faces to the aqueous solutions on either side. The strict linearity of the Scatchard plot (Fig. 2 *B*) in uncharged bilayers down to ionic strengths as low as 12.5 mM—i.e., the failure to observe a local potential effect—argues that the effective charge density at the channel mouth contributed by the protein is lower than one charge per 50 nm^2. Since the cross-section of the channel's entryway has been estimated to be ~1 nm^2 (Miller, 1982; Coronado and Miller, 1982), we suggest that there are essentially no net fixed charges lining the mouth of the SR K^+ channel.

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DISCUSSION

Session Chairman: Alan Finkelstein *Scribes*: Alice Chu and Sarah S. Garber

MONTAL: How do you select the orientation of the potassium channel in the lipid bilayer?

MILLER: As a conservative estimate, the vesicles are right-side out 90% of the time. The ATP catalytic site of the Ca^{++} ATPase is facing the outside of the vesicle after the homogenization of the muscle. Fusion of the vesicles to the bilayer is an orientation preserving process.

MONTAL: If the fitting of the plots (Fig. 2) was done in a more rigorous fashion, would it be possible to find two orientations of the channels?

MILLER: No. We are looking at single channel records so the orientation of the channel is always apparent. Over 90% of the channels in the bilayer have the same orientation. We haven't looked at macroscopic data.

COHEN: I agree with your interpretation that the channel mouth protrudes 10 or 20 Å from the membrane surface. Rather than protruding into the aqueous phase, it equally possible that the mouth could be 10–20 Å inside the membrane. This would be consistent with the idea that the relevant "mouth" for potassium conduction is at the entrance to the selectivity region of the channel. If this were the case, the constraint that both "mouths" sense equal electrostatic potentials would place the constriction region exactly in the middle of the membrane. Could you comment on this?

MILLER: I like the idea that the channel has a short constriction with a wide mouth on each side of the membrane. There is no reason to presume that the channel is sticking out into the aqueous phase. The ion selective area could be inside the channel, isolated from the bulk surface.

COHEN: With regard to the possible physiological role of the channel as an electrical shunt during Ca^{++} uptake or release from SR, it would seem that this mechanism could work only during Ca^{++} release from sarcoplasmic reticulum, not during Ca^{++} uptake. The channel is opened by positive voltage on the *cis* side of the bilayer, which corresponds to the outside of the SR vesicles and to the cytoplasmic side of the SR in vivo. This side should become positive relative to the lumenal side during Ca^{++} release and negative during Ca^{++} uptake. Therefore the channel should be closed during uptake and open during Ca^{++} release. Could you comment on this?

MILLER: I see your point. We have no direct evidence that the channel behaves in vivo as it does in the bilayer, because the voltage dependence of the channel in the bilayer. However, the voltage dependence is very sluggish.

FINKELSTEIN: The point was made in the paper that the qualitative effect of changing the lipids was consistent with an interpretation of a change in the surface charge. Quantitatively, you then went on to propose a geometrical interpretation of why the channel does not feel the full effect of the surface potential. Could you propose a model where the channel mouth is isolated from the full surface charge by an uncharged buffer zone in the plane of the membrane? Have you considered trying to calculate the surface charge at the mouth of channel to fit this type of model?

MILLER: We have considered this type of lateral isolation model. We have not tried to make any analytical predictions for these models.

PARSEGIAN: One could spend many graduate student theses on calculations of this type. The important point is not to be very serious about the geometric alibi you happen to choose. There are many models that would give you the correction you need.

RUBINSON: Could you give me an idea of the mixing time of the lipids of the SR vesicle with the known lipids of the bilayer? Do you think there is a difference in the mixing of the lipid near the channel protein with those outside of it?

MILLER: Given the size of a single SR vesicle (0.1 μm), the equilibrium mixing time of the lipids is on the order of milliseconds. The incorporation of a single SR vesicle into the bilayer would change the bilayer composition by a factor of 10^{-5}, so we don't think this is a problem.

BLANK: You are making measurements on discrete systems of single channels. But the surface charge density you speak of is an average concept. In 1968, Israel Miller and I (*J. Colloid Interface Sci.* 26:26–40) found that the movement of cupric ions through a positively charged monolayer at a mercury/water interface was a function of the surface charge. Ours was an average measurement and the rate of ion movement could be explained on the basis of electrical double layer theory. Do your discrete measurements depend on the surface charge and the ionic strength in the same way? Should you use another term in place of the macroscopic term surface charge density?

MILLER: We are using an average, smeared out surface charge density, as treated in the Gouy-Chapman theory.

JAKOBSSON: A gating mechanism does not sense a potential, it senses an electric field. What is the change in the electric field imposed by the change of the surface charge when you change the phospholipids? This would change the physical interpretation of the model.

MILLER: This is not a paper about gating. It is about conduction.

JAKOBSSON: The principle is the same. What is the change in the electric field, at the critical place in the channel, as a function of surface charge?

MILLER: I can't say.

HALL: Neutral membranes have a dipole potential. It might be possible to distinguish whether the channel is in the water or in the membrane by varying the dipole potential. Have you looked at this?

MILLER: That's a good point. How would you systematically vary the dipole potential?

FINKELSTEIN: An easy experiment to test this would be to add phloretin to one side of the membrane.

LABARCA: Increasing the proton concentration does change the conductance of the channel. Have you considered this complication?

MILLER: Given the effective rise in potassium concentration that we can calculate, the proton concentration should increase with the potassium concentration at the surface of the membrane. Protons are competitive with potassium (the pK of the channel is 5). We have been careful to keep out of the concentration range where protons and potassium are competitive. The experiments were done at pH 7.5.

DILGER: Have you considered using a charged amphiphile to change the surface charge? The amphiphile may adsorb to the protein-water interface as well as the lipid-water interface. Could this be used to distinguish the geometrical models?

MILLER: Do you want me to put detergents into my bilayer?! Do you mean stearoylamine for example? No, we have not done this, because we were worried that the large cations would block the channel. No cations have been found that do not affect the channel conductance. We used cationic phospholipids to rule out the possiblity that we were seeing a decrease in the conductance due to blocking of the channel instead of due to a decrease in the surface potential.

ZIMMERBERG: If the mixing of the lipids around the channel is slower then free diffusion, you might expect to see a slower relaxation of the channel conductance. Did you ever see anything like this?

MILLER: I haven't seen such a relaxation on the timescale of 2 ms.

IWASA: Is it possible, using this incorporation procedure, that you are selecting for a population of channel based on the phospholipid structure?

MILLER: This is important when only one or two vesicles of a population of vesicles on the order of 10^{12} is incorporated into the bilayer. That is why we were so careful to check the maximum conductances, permeability ratios, selectivities, and blocker kinetics of the channels. By those criteria we are working with the same channel in a phosphatidylserine bilayer as we are in a phosphatidylethanolamine bilayer.

RUBINSON: Is it possible to maintain the ionic strength while changing the potassium concentration?

MILLER: No. You need an inert cation that does not interact with the channel at all to do those experiments. No inert cations have been found in this system. All cations change the conduction of the channel in some way.

RUBINSON: Are the lipid phases of the SR and the bilayer miscible?

MILLER: Yes, they ought to be, especially since the lipids of the SR are at an infinite dilution.

RUBINSON: Is there a phase diagram for such systems?

MILLER: No, but the fact that a lipid effect is observed says that the lipids must mix to some extent.

SZABO: Did you have any Ca^{++} present?

MILLER: All experiments were done in the presence of EDTA. Otherwise these experiments would be invalid.

SZABO: There is a danger that multivalent cations could screen the surface charge from the channel, giving rise to an interpretation that the channel does not see the full surface charge, when in fact it may.

Experiments such as these have been done on gramicidin, where the mouth of the channel is near the surface of the membrane and the channel feels the entire surface potential. This method could be used to measure the size of the channel mouth. At low ionic strength, when the Debye length is very large, you expect the exposed double layers to overlap and the charge to be smeared out. In this case, the Gouy-Chapman theory is applicable. This is what one sees in the gramicidin channel. As you increase the ionic strength, the Debye length becomes very small, ~3 Å. Under these circumstances, there would be deviations from the theory. This could be a nice empirical measure of the width of the channel mouth. For gramicidin, the theory works beautifully up to 0.1 M, beyond which the experiments have not been done properly.

As for dipole potentials, these are sensed by the gramicidin channel. Channel conductance changes for lipids having large differences in dipole potential. However the gramicidin A channel sees only 10–20% of the dipole potential.

FINKELSTEIN: Aren't divalent cations present during the actual fusion of the vesicles to the bilayer, and weren't these ions removed after the event?

MILLER: Yes.

ANDERSEN: Gramicidin channels see much more than 10–20% of the dipole potential.The reason you do not see a larger conductance when you add phloretin is that you change the dipole potential at a place which is not a rate-limiting barrier. The dipole potential can thus be changed by a large amount, and this may not change the conductance very much. Dr. Heitz, Dr. Mazet and I have done experiments with gramicidin M (Heitz, F., Spach, and Trudelle. 1982. *Biophys. J.* 40:87–89), which appears to have a high central barrier. In this case phloretin will cause a large change in the conductance because the dipole potential was changed at a point where there was a major resistance to ion movement.

SZABO: What I was referring to was dipoles at the lipid-water interface. It is pretty clear that the gramicidin channels start inside the lipid-water interface.